Respiratory Syncytial Virus Binds and Undergoes Transcription in Neutrophils From the Blood and Airways of Infants With Severe Bronchiolitis

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Background. Neutrophils are the predominant cell in the lung inflammatory infiltrate of infants with respiratory syncytial virus (RSV) bronchiolitis. Although it has previously been shown that neutrophils from both blood and bronchoalveolar lavage (BAL) are activated, little is understood about their role in response to RSV infection. This study investigated whether RSV proteins and mRNA are present in neutrophils from blood and BAL of infected infants.

Methods. We obtained blood and BAL samples from 20 infants with severe RSV bronchiolitis and 8 healthy control infants. Neutrophil RSV F, G, and N proteins, RSV N genomic RNA, and messenger RNA (mRNA) were quantified.

Results. RSV proteins were found in BAL and blood neutrophils in infants with RSV disease but not in neutrophils from healthy infants. BAL and blood neutrophils from infants with RSV disease, but not those from healthy infants, expressed RSV N genomic RNA, indicating uptake of whole virus; 17 of 20 BAL and 8 of 9 blood neutrophils from patients expressed RSV N mRNA.

Conclusions. This work shows, for the first time, the presence of RSV proteins and mRNA transcripts within BAL and blood neutrophils from infants with severe RSV bronchiolitis.

Bronchiolitis is the commonest cause of lower respiratory tract infection in children under the age of 1 year [1]. Most cases are caused by respiratory syncytial virus (RSV), of which there are 2 serotypes, A and B. Serotype A is the most common serotype found in United Kingdom (UK) seasonal epidemics, although proportions vary annually [2]. The clinical manifestations of RSV infection range from mild upper respiratory tract symptoms, to severe lower respiratory tract infection presenting as low-grade fever that can progress over several days to coughing, wheezing, shortness of breath, and difficulty with feeding. Although some infants with bronchiolitis have clinical symptoms beyond the lungs including otitis media, myocardial failure, seizures, and hepatitis, [3] whether these extrapulmonary manifestations are due to RSV infection itself or to secondary effects is not fully understood. RSV RNA has been detected outside the lungs in cerebrospinal fluid [4], myocardium [5], and liver [6].

Human RSV is transmitted by inhalation of aerosolized virus droplets to the airway epithelium [7], a major site for viral replication. Infection is followed by cytokine release and airway inflammation [8, 9]. The surface glycoproteins RSV F and G facilitate binding of the virus [10, 11] to their target cells whereas RSV N protein is located inside the virion, tightly bound to the genome. In vitro, RSV has been shown to infect epithelial cells [12]), alveolar macrophages [13], dendritic cells [14, 15], and bone marrow stromal cells [16]. Infection of the epithelial cells promotes the release of

Table 1. Characteristics of Patients and BAL and Blood Samples

	Bronchiolitis infants	Control infants
Clinical information		
No. of infants (male)	20 (10)	8 (5)
No. preterm (male)	11 (5)	0
Age on admission, corrected for prematurity, in wk median (range)	10 (–5, 20)	12 (6, 20)
Weight on admission, kg mean (SEM)	3.8 (.45)	4.3 (2.0)
Gestation at birth, wk median (range)	38 (27, 42)	40 (37, 40)
BAL characteristics before enrichment		
% viable cells mean (SEM)	95 (2.5)	95 (1)
Total cell count/mL mean (SEM)	1.85 (.7)	1.5 (.9)
% neutrophils median (IQR)	84 (2.5)	37 (3)
% alveolar macrophages median (IQR)	12.5 (1.5)	47 (3)
% lymphocytes median (IQR)	5 (.5)	7 (3)
BAL characteristics after enrichment using Polymorphprep		
% viable cells mean (SEM)	93 (2)	94 (2)
% neutrophils median (IQR)	95 (2.5)	96 (2)
Blood characteristics before enrichment		
% viable cells mean (SEM)	97 (2)	96 (1)
Neutrophils × 10^9/L mean (SEM)	4.93 (1.46)	4.5 (1.56)
Monocytes × 10^9/L mean (SEM)	0.99 (.15)	1.0 (.18)
Lymphocytes × 10^9/L mean (SEM)	3.67 (.3)	3.63 (.35)
Eosinophils × 10^9/L mean (SEM)	0.088 (.04)	0.082 (.045)
Blood characteristics after enrichment		
% viable cells mean (SEM)	95 (2)	94 (1.5)
% neutrophils median (IQR)	96 (1.5)	95 (1)

NOTE. BAL, bronchoalveolar lavage; IQR, interquartile range; SEM, standard error of mean.

cytokines [17–19] and the recruitment of inflammatory cells [17]. Neutrophils are recruited early in the course of infection and represent >80% of the inflammatory cell infiltrate in bronchoalveolar lavage (BAL) at the peak of the patient's clinical symptoms [17]. Neutrophils in the airways are highly activated [20], release cytokines [21–24], and have delayed apoptosis [25, 26]. However, how they influence RSV clearance or viral replication within the human airway remains largely unknown.

The aim of this study was to determine whether RSV directly interacts with, or indeed infects, infant neutrophils in the lung and peripheral circulation, by measuring RSV protein and gene transcript expression in neutrophils from the blood and BAL of infants with severe RSV bronchiolitis.

PATIENTS AND METHODS

Study Population, Sample Collection, and Preparation
We recruited 20 infants who were aged <18 months and admitted with severe RSV bronchiolitis to the intensive care unit at Alder Hey Children's Hospital. RSV bronchiolitis was confirmed by direct immunofluorescence of nasopharyngeal aspirates. The control group consisted of 8 uninfected healthy infants of comparable age, ventilated prior to elective noncardiac surgery. Infants with underlying cardiorespiratory disease were excluded.

Ages were corrected for prematurity. Blood and BAL samples were collected and prepared as described in previous studies [17, 18, 20] as soon as possible after intubation to minimize the effects of mechanical ventilation on the airways (Table 1). The Liverpool Paediatric Research Ethics Committee approved the study and parents/guardians of participants gave full written consent for study procedures.

Enrichment of BAL and Preparation of Blood Neutrophils
As described previously [20], BAL was filtered through a 60-μm pore size gauze (Sefar Nitex 03-48/31) to remove bulk mucous and then centrifuged. The supernatant was removed and cells were resuspended in RPMI 1640. Neutrophils were then enriched using a sedimentation gradient of Polymorphprep (Axis-Shield PoC AS Norway) as directed by the manufacturer. After centrifugation, cells were resuspended and washed in RPMI 1640 prior to staining. Blood samples were collected into heparinized tubes and blood neutrophils prepared as stated above on a Polymorphprep sedimentation gradient prior to analysis.

FACS Analysis: Protein Expression of RSV F, G, and N
For total expression of RSV, F, G, and N neutrophils were permeabilized, fixed (BD Cytofix and Cytoperm, BD Biosciences), and labeled. For cell surface expression only, cells were

stained without prior permeabilization. Cells were triple stained, initially using a primary test antibody, RSV F, G, or N (Acris Antibodies), or isotype-matched control followed by a secondary antimouse fluorescein isothiocyanate (FITC) secondary antibody, a neutrophil marker (phycoerythrin [PE]–labeled myeloperoxidase [MPO] or cluster density [CD] 16 separately), and ethidium monoazide bromide (EMA; Biotium) to allow exclusion of nonviable cells. Samples were analyzed on a FACSort flow cytometer (Becton Dickinson) collecting a minimum of 10 000 events. All samples were analyzed with the same voltage and gain settings during the study period. Calibration beads were used to control for laser decay. The isotype control was set in the first decade to maximize sensitivity of samples. Cells were deemed positive for RSV proteins if their fluorescence was greater than the 95th percentile of the fluorescence of the matched isotype control. Positive values were calculated in each case by subtracting the isotype control values, typically less than 1%, from the observed test values. Data were processed using WinMDI software, version 2.9. Neutrophils were gated by size, granularity, viability, and CD16 or MPO expression.

Cellular Localization of RSV Protein by Immunocytochemistry

Neutrophils were resuspended in 4% sucrose/paraformaldehyde and air-dried onto slides. Slides were then stained for RSV proteins using F, G, or N primary monoclonal antibodies, or isotype-matched control antibodies, followed by a PE-labeled secondary goat antimouse antibody and with the nuclear stain DAPI. The slides were fixed and visualized by fluorescence microscopy at 40× magnification. The RSV-infected Hep2 (multiplicity of infection, 0.25) cell line was used as a positive control.

Real-Time-Polymerase Chain Reaction Quantification of RSV N Genomic RNA and Messenger RNA

Genomic RNA and messenger RNA (mRNA) were measured using Real-Time-Polymerase Chain Reaction (RT-PCR) and Taqman primers for either RSV N serotype A or RSV N serotype B (Primer Design; catalog numbers Path-RSVA-std, Path-RSVB-std, respectively). All PCR reactions were in triplicate using an Applied Biosystems 7300 system and standardized to the housekeeping gene L32 using the ΔCt method to give the median logfold ratio. RNA from an RSV-infected Hep2 cell line was used as a positive control and water was used as a negative control.

Statistical Analysis

RSV protein (F, G, and N) and mRNA (N gene) expression was studied in enriched neutrophil blood and BAL samples from infants with RSV bronchiolitis and the control group. Data were analyzed using independent samples t tests or Mann-Whitney U tests depending on data distribution determined by the 1-sample Kolmogorov-Smirnov test. Differences in RSV protein (F, G, and N) and mRNA (N gene) expression between blood and BAL within participants from each patient group were examined using paired t tests. Data were analyzed using SPSS 17 software (SPSS Inc).

RESULTS

RSV F, G, and N Proteins Are Expressed Both on and in BAL and Blood Neutrophils From Infants With RSV Bronchiolitis

All samples from infants with RSV disease expressed RSV proteins F, G, and N on both BAL and blood neutrophils. Figure 1A shows representative histograms for the total and cell surface expression of RSV proteins on BAL and blood neutrophils from an infant with RSV disease and a healthy control. RSV F, G, and N proteins were not expressed on BAL or blood samples from healthy controls. For BAL neutrophils from RSV-infected infants, the mean percentages (±SEM) of neutrophils positive for RSV F, G, and N were 86.6 (2.8), 82.9 (3.2), and 83.8 (2.9), respectively, with a median (interquartile range [IQR]) mean fluorescent intensity (MFI) compared with isotype controls for RSV F, G, and N proteins of 56.9 (54.7), 52.0 (44.4), and 66.4 (37.6), respectively. For blood neutrophils from RSV-infected infants, the mean percentages (SEM) of neutrophils positive for RSV F, G, and N were 55.7 (2.9), 54.8 (4.7), and 54.0 (4.9), with a median (IQR) MFI compared with isotype controls for RSV F, G, and N proteins of 29.2 (59.5), 26.8 (50.0), and 27.4 (46.2), respectively (Figures 1B and 1C). A significantly greater proportion of the BAL neutrophils from RSV-infected infants expressed RSV proteins F, G, and N ($P < .001$) compared with blood neutrophils, and the levels of expression of these RSV proteins in positive cells (as indicated by the MFI of the positive cells) was significantly different between BAL and blood ($P = .013$ and .012 for proteins G and N, respectively, but not for RSV F protein [$P = .079$]). This indicates that both the proportion of neutrophils expressing RSV proteins and the level of expression of these proteins were greater in the lung than in the blood.

Because our monoclonal antibodies for FACS analysis were not directly conjugated, it was not possible to determine the absolute numbers of antibody binding sites per neutrophil for each of the proteins. In order to gain some insight into the stoichiometry of expression of RSV proteins F, G, and N in neutrophils, the analysis shown in Figure 2 was performed. These data show very strong correlations between the expression of these individual proteins in both BAL and blood neutrophils (all $P < .001$). These data thus demonstrate that levels of expression of all 3 viral proteins were proportionate to each other, strongly indicating stoichiometric expression. This relationship held when analyzing both the numbers of positive cells (% positive) or the levels of expression (MFI).

Immunofluorescence was used to confirm the expression of RSV proteins in neutrophils compared with RSV-infected Hep2 cells, which were used as a positive control to assess the
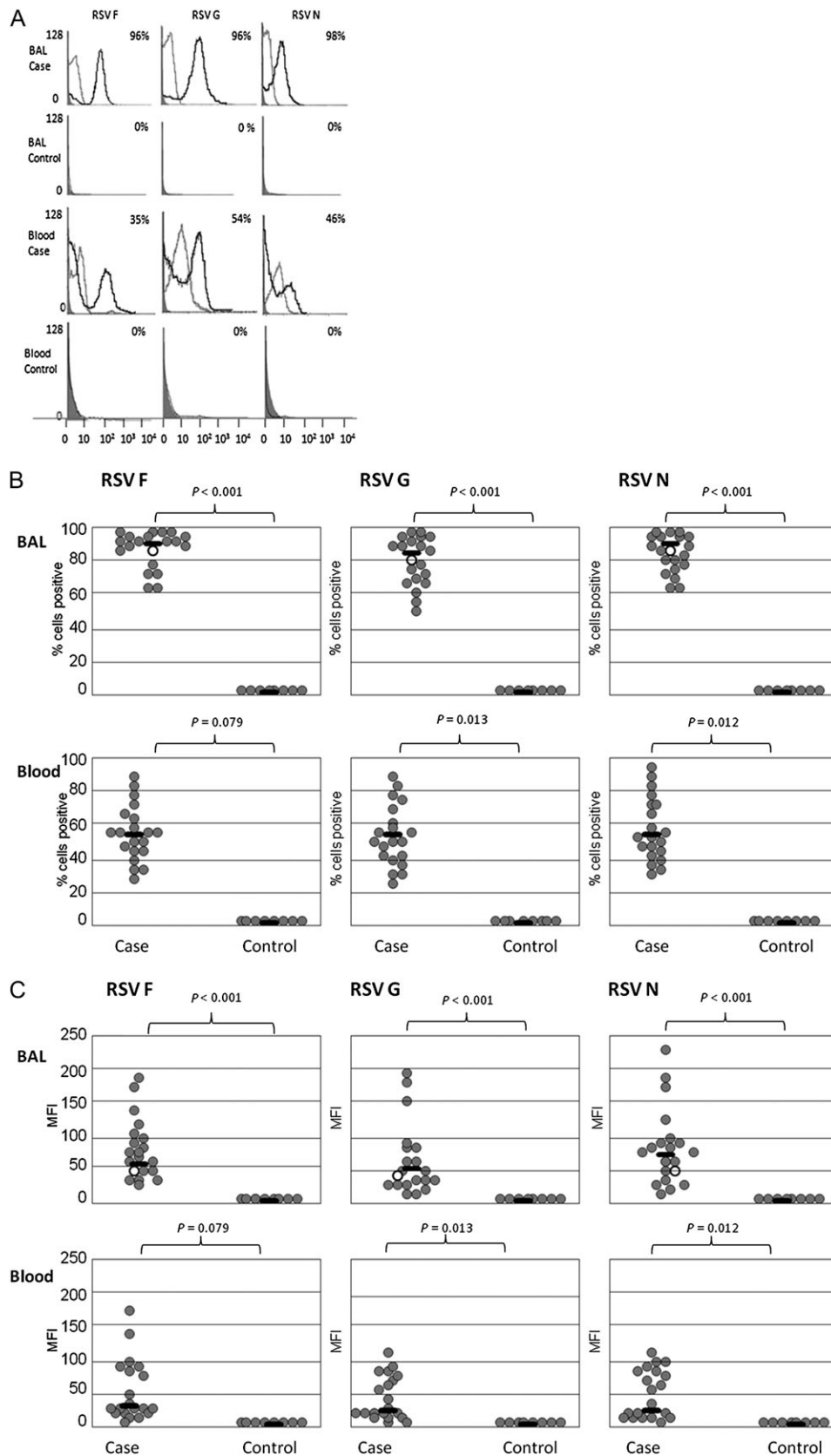
Figure 1. Respiratory syncytial virus (RSV) F, G, and N protein expression by bronchoalveolar lavage (BAL) and blood neutrophils from patient and control samples. *A,* Flow cytometry analysis of total (black line) and cell surface expression only (gray line) of RSV protein binding to BAL or blood
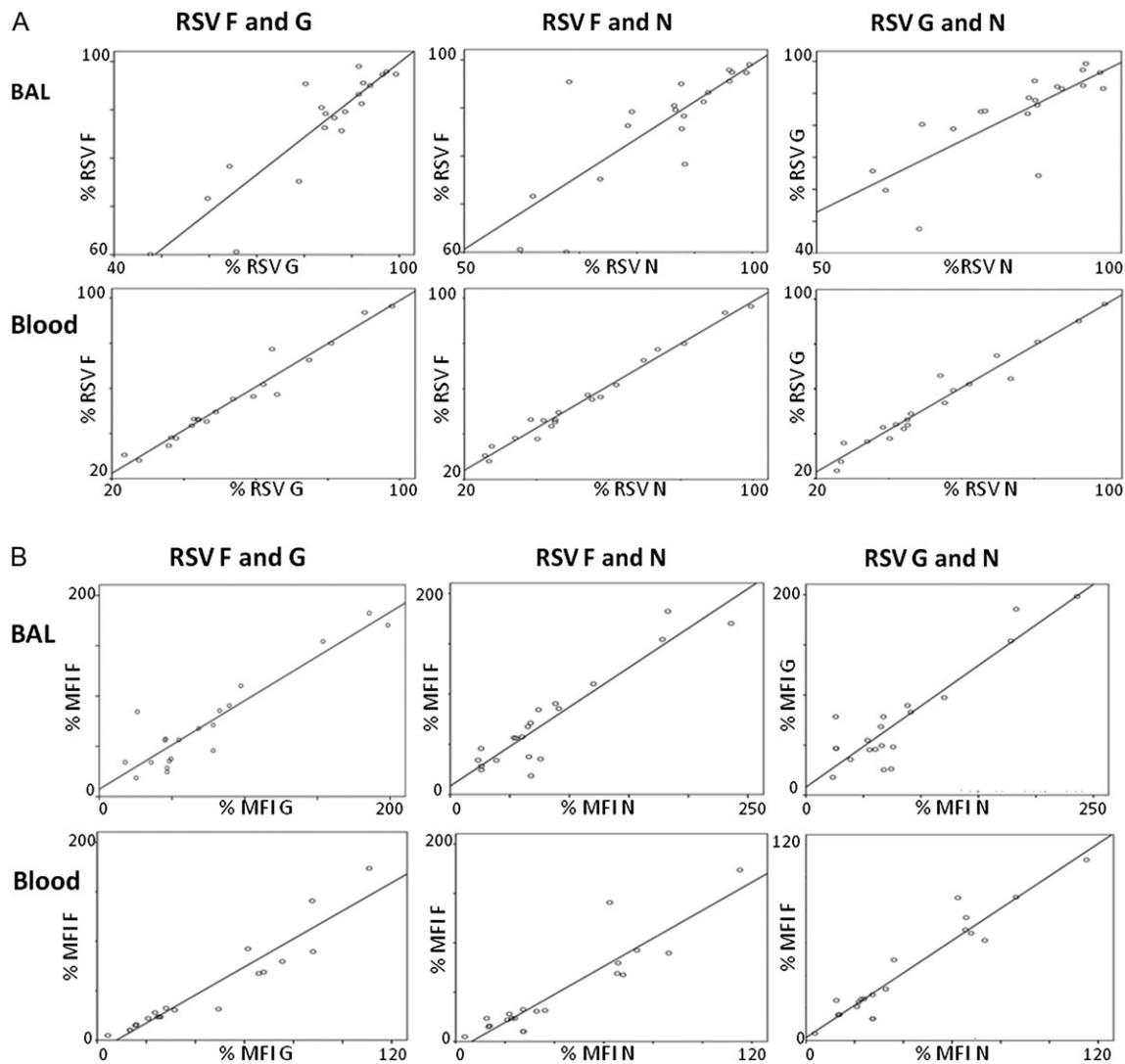
Figure 2. Correlation between expression of different RSV F, G, and N protein levels in BAL and blood neutrophils. *A*, Correlation in percentage of cells positive between F (y-axis) and G (x-axis) (BAL $r^2 = .899$, $P < .001$; blood $r^2 = .96$, $P < .001$); F (y-axis) and N (x-axis) (BAL $r^2 = .835$, $P < .001$; blood $r^2 = .982$, $P < .001$) and G (y-axis) and N (x-axis) (BAL $r^2 = .911$, $P < .001$; blood $r^2 = .957$, $P < .001$). Each point represents an individual sample value. *B*, Correlation in mean fluorescence intensity (MFI) between F (y-axis) and G (x-axis) (BAL $r^2 = 0.86$, $P < .001$; blood $r^2 = .899$, $P < .001$); F (y-axis) and N (x-axis) (BAL $r^2 = .86$, $P < .001$; blood $r^2 = .86$, $P < .001$) and G (y-axis) and N (x-axis) (BAL $r^2 = .83$, $P < .001$; blood $r^2 = .911$, $P < .001$). Each point represents an individual sample.

specificity of the antibodies. In non–RSV-infected Hep2 cells, no positive staining was observed for RSV proteins F, G, or N (data not shown), whereas strong positive staining was observed in these cultured cells infected with RSV in vitro (Figure 3). BAL patient neutrophils stained strongly for RSV F, G, and N proteins (Figure 3).

RSV Genomic RNA and mRNA Is Detected in BAL and Blood Neutrophils From Infants With RSV Bronchiolitis
PCR analysis was carried out after flow cytometry on 20 of 20 BAL and 9 of 20 blood neutrophil samples from RSV-positive infants, and 8 of 8 BAL and 5 of 8 blood neutrophil samples from control infants (Table 2).

neutrophils from infants with and without RSV infection. Representative histograms are shown after gating for viable cells by ethidium monoazide bromide staining, CD16 (a neutrophil marker), and respective anti-RSV monoclonals or isotype controls (shown shaded in gray). Numbers shown represent the percentage of positive cells above isotype control values following permeabilization. *B*, Summary data for percentage of positive cells for each patient ($n = 20$) or controls ($n = 8$). Each point represents an individual value, while the bar represents the sample mean. *C*, Summary data for mean fluorescence intensity of each sample analyzed by FACS. Each point represents an individual sample value. The bar represents the median value. *B* and *C*, the open dot highlights the corresponding data for the RSV-positive infant BAL neutrophils shown in Figure 3.
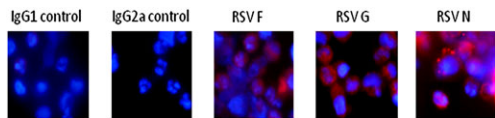
Figure 3. Detection of RSV F, G, and N proteins within the cytoplasm of neutrophils by immunofluorescence. A representative BAL sample (shown in Figures 1B and 1C by an open dot with percentage of positive cells F, G, and N [87, 80, 86, respectively] and MFI for F, G, and N [50, 43, 50, respectively]) was stained with isotype control (IgG1 and IgG2a) or RSV-specific F, G, and N monoclonal antibodies. Positive staining (PE) is shown in red while nuclei that were counterstained by DAPI are shown in blue.

Twenty of 20 BAL and 9 of 9 blood neutrophil samples from RSV-positive infants and no BAL or blood neutrophil samples from control infants expressed genomic RSV N (serotype A). Furthermore, 17 of 20 BAL neutrophils and 8 of 9 blood neutrophil samples from RSV-positive infants also expressed RSV N mRNA (serotype A). For the RSV-positive infants, there was no significant difference in the levels of expression of mRNA for RSV N (serotype A) compared with the housekeeping gene L32 between the blood and BAL compartments ($P = .248$); the median logfold ratios of RSV N mRNA to L32 mRNA ($\pm$IQR) were blood .2214 ($\pm$8.047) and BAL .814 ($\pm$14.185). Two paired blood and BAL samples showed the presence of both RSV N mRNA (serotype A and serotype B). No healthy control samples were positive for genomic RNA or RSV N mRNA (serotype B) (Table 2). DNA sequencing of PCR products generated using RSV F, G, and NS1 primers confirmed the identity of the products as the RSV gene under study (data not shown).

DISCUSSION

This is, to our knowledge, the first clinical study to investigate whether RSV interacts with neutrophils in infants with RSV bronchiolitis. We show that RSV F, G, and N proteins are present within the neutrophils from the airways of these infants

Table 2. Summary RT-PCR Data for RSV Genomic and mRNA for RSV N

RT-PCR RSV N; A and B serotypes	Cases		Controls	
	Blood	BAL	Blood	BAL
Genomic RSV A	9/9	20/20	0/5	0/8
mRNA RSV A	8/9	17/20	0/5	0/8
Genomic RSV B	2/9[a]	2/20[a]	0/5	0/8
mRNA RSV B	2/9[a]	2/20[a]	0/5	0/8

NOTE. BAL, bronchoalveolar lavage; RSV, respiratory syncytial virus; RT-PCR, real-time polymerase chain reaction.

[a] Two of 20 RSV-positive infants expressed both RSV N gene genomic RNA and messenger RNA for both serotypes A and B in both blood and BAL neutrophils, indicating dual infection.

and make the novel and potentially important discovery that these proteins are also expressed in the blood neutrophils of these patients. Expression was determined by measuring protein levels but also by measuring the levels of expression of the genes. Furthermore, the presence of RSV mRNA in neutrophils indicates that active transcription of the virus is likely to be occurring.

Neutrophils are phagocytic cells and so it might be predicted that viral proteins would be present within them. For example, viral proteins could be present within neutrophils following phagocytosis of virus-infected cells, possibly infected epithelial cells or other neutrophils. Phagocytosis of apoptotic neutrophils by other neutrophils has been demonstrated in vitro, and contact with apoptotic cells inhibited the proinflammatory function of neutrophils, suggesting that this process contributes to the resolution of inflammation [27]. However, certain aspects of our findings suggest that phagocytosis by neutrophils is not the explanation. The pattern of viral protein expression was of diffuse cytoplasmic staining (Figure 3) rather than being localized within phagocytic vesicles. If the viral particles entered by phagocytosis into the denaturing, hostile environment of the phagocytic vacuole, it is unlikely that we would have detected any labile viral mRNA intact within neutrophils.

Our data show a strong correlation between the expression of RSV F, G, and N proteins in neutrophils, which is indicative of their stoichiometric expression. This suggests that intact viral particles are present within neutrophils. These particles could arise from uptake of whole virus by neutrophils, or intriguingly, via replication of virus within the neutrophil. We have no direct evidence for the latter possibility, but it is of note that we detected RSV mRNA in both blood and BAL neutrophils. Although this finding is suggestive of viral transcription, it does not necessarily imply whole viral replication. The synthesis of new viral particles requires the expression of antigenome, which is only believed to occur transiently at very low levels during viral replication [28]. Furthermore, active transcription could also represent abortive replication. Experiments in airway epithelial cells from the cotton rat have shown that RSV is capable of abortive replication, characterized by transcription of individual viral genes but no synthesis of viral progeny [29]. In this case, RSV transcripts and protein accumulated and resulted in an increased inflammatory response [30]. We can speculate that if abortive replication of RSV occurs in neutrophils, it may have 2 important effects that will be detrimental to the host: first, to delay neutrophil apoptosis and therefore prolong RSV survival [25, 26]; and second, to exacerbate lung damage by acting as a proinflammatory stimulus for the release of cytokines and chemokines.

A surprising and unexpected finding was the discovery that blood neutrophils from RSV-infected infants expressed RSV viral proteins and both genomic RNA and mRNA for RSV. In human RSV infection, RSV RNA has been demonstrated in

whole blood [31], and RSV antigens [32] and RNA have been found in circulating blood mononuclear leukocytes [33, 34] but not, until now, in either blood or lung neutrophils. RSV has also been demonstrated within the blood in a murine model of RSV airway infection. In these experiments the time course of RSV RNA detected in the blood was reported to mirror viral replication in the respiratory tract and peak disease severity [35]. It is noteworthy that the RSV-infected infants in our study had severe bronchiolitis, requiring hospitalization in intensive care and ventilation. It would be interesting now to determine if neutrophils from the blood of RSV-infected infants with less severe bronchiolitis also express RSV proteins and RNA, or whether this is restricted to the more severe cases.

Our novel findings raise an extremely important and as yet unresolved question: How does RSV, or its proteins and mRNA transcripts, enter blood neutrophils? If it is assumed that the virus initially enters the nasal epithelium via inhalation of virus-containing droplets, then there are a number of possibilities. First, viral particles may be released from infected airway epithelial cells and then enter the circulation, where they are taken up by circulating neutrophils. Second, neutrophils may take up the virus in the airway lumen and then recirculate back into the bloodstream. Similar mechanisms for viral infection of the respiratory tract and subsequent appearance in the circulation have been proposed for influenza. As with RSV infection, H5N1 (avian influenza) infection is primarily localized to the respiratory tract but also can be found at sites outside the lung in severe disease [36]. Recent in vitro experiments have shown that H5N1 can be secreted by human lung microvascular endothelial cells, which in vivo would lead to secretion into the systemic circulation [37]. We have no direct evidence that RSV-infected neutrophils transmigrate from the lung back into the circulation; however, there is evidence in the literature that such a process can occur in vivo. Buckley et al [38] showed that neutrophils can reverse-transmigrate across an endothelial monolayer, that is, initially move though a monolayer and then migrate back. These reverse-transmigrated cells are characterized by distinctive cell surface marker expression (CD54^{high}, CXCR1^{low}). Cells with this phenotype were identified during reverse-migration experiments in vitro and were later found to be present in the bloodstream of healthy individuals in low numbers but at higher numbers in patients with systemic inflammation. It would therefore be interesting to determine if the RSV-positive neutrophils in RSV-infected infants are also CD54^{high}, CXCR1^{low}.

The mechanism by which RSV may enter neutrophils is unknown. RSV F protein has been shown in vitro to be a ligand for Toll-like receptor 4 (TLR4) on peripheral blood macrophages [13]. We have recently reported decreased total TLR4 protein in both blood and BAL neutrophils from infants with severe RSV bronchiolitis. The decrease in total TLR4 was significantly greater in those infants born at full term compared with those born before 37 weeks (preterm infants) [20]. Both groups with RSV infection had significantly less total TLR4 than control healthy infants. For each infant with RSV infection, BAL and blood total neutrophil TLR4 indirectly correlated with the percentage of BAL and blood neutrophils positive (respectively) for each of the 3 RSV proteins F, G, and N. In vitro experiments showed that the decrease in total neutrophil TLR4 was not due to competitive binding. We speculate that neutrophil TLR4 RSV interaction may play an important role in the pathogenesis of severe RSV disease, especially in those infants born at full term.

In summary we have shown that neutrophils in the BAL and blood of infants with severe RSV infection take up RSV, which may then undergo active transcription. This evidence of direct interaction between RSV and neutrophils in the airway and peripheral circulation of infants with severe RSV bronchiolitis provides fundamentally important insights into the role of inflammatory cells recruited to the airways during infections with respiratory viruses.

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References

1. Smyth RL, Openshaw PJM. Bronchiolitis. Lancet **2006**; 368,312–22.
2. Anderson LJ, Hierholzer JC, Tsou C, et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis **1985**; 151:626–33.
3. Eisenhut M. Extrapulmonary manifestations of severe RSV bronchiolitis. Lancet **2006**; 368:988.
4. Zlateva KT, Van Ranst M. Detection of subgroup B respiratory syncytial virus in the cerebrospinal fluid of a patient with respiratory syncytial virus pneumonia. Pediatr Infect Dis J **2004**; 23:1065–6.
5. Fishaut M, Tubergen D, McIntosh K. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J Pediatr **1980**; 96:179–86.
6. Nadal D, Wunderli W, Meurmann O, Briner J, Hirsig J. Isolation of respiratory syncytial virus from liver tissue and extrahepatic biliary atresia material. Scand J Infect Dis **1990**; 22:91–3.
7. Hall CB, Douglas RG Jr, Geimann JM. Quantative shedding of RSV. J Infect Dis **1975**; 132:151–6.
8. Hall CB, Douglas RG Jr, Geimann JM. RSV quantification and duration of shedding. J Pediatr **1979**; 89:1–15.
9. Openshaw PJ. Antiviral immune responses and lung inflammation after respiratory syncytial virus infection. Proc Am Thorac Soc **2005**; 2:121–5.
10. Levine S, Klaiber-Franco R, Paradiso PR. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J Gen Virol **1987**; 68:2521–4.
11. Walsh EE, Hruska J. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J Virol **1983**; 47:171–7.

12. Mastronarde JG, Monick MM, Gross TJ, Hunninghake GW. Amiloride inhibits cytokine production in epithelium infected with respiratory syncytial virus. Am J Physiol 1996; 271:L201–7.

13. Kurt-Jones EA, Popova L, Kwinn L, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 2000; 1:398–401.

14. Cirino NM, Panuska JR, Villani A, et al. Restricted replication of respiratory syncytial virus in human alveolar macrophages. J Gen Virol 1993; 74(Pt 8):1527–37.

15. Jones A, Morton I, Hobson L, Evans GS, Everard ML. Differentiation and immune function of human dendritic cells following infection by respiratory syncytial virus. Clin Exp Immunol 2006; 143:513–22.

16. Rezaee F, Gibson LF, Piktel D, Othumpangat S, Piedimonte G. Respiratory syncytial virus infection in human bone marrow stromal cells. [published online ahead of print October 22, 2010]. Am J Respir Cell Mol Biol.

17. McNamara PS, Selby A, Hart CA, Smyth RL. Bronchoalveolar cellularity in infants with severe RSV bronchiolitis. Arch Dis Child 2003; 88:922–6.

18. McNamara PS, Selby A, Hart CA, Smyth RL. Pro- and anti-inflammatory responses in RSV bronchiolitis. Eur Respir J 2004; 23:106–12.

19. McNamara PS, Flanagan BF, Hart CA, Smyth RL. Production of chemokines in the lungs of infants with severe RSV bronchiolitis. J Infect Dis 2005; 191:1225–32.

20. Halfhide CP, Flanagan BF, Hunt JA, et al. Neutrophil TLR4 expression is reduced in the airways of infants with severe bronchiolitis. Thorax 2009; 64:798–805.

21. McNamara PS, Baldwin LM, Newland P, Hart CA, Smyth RL. Interleukin 9 production in the lungs of infants with severe respiratory syncytial virus bronchiolitis. Eur Respir J 2004; 20:651–7.

22. Jaovisidha P, Peeples ME, Brees AA, Carpenter LR, Moy JN. Respiratory syncytial virus stimulates neutrophil degranulation and chemokine release. J Immunol 1999; 163:2816–20.

23. Bataki EL, Evans GS, Everard ML. Respiratory syncytial virus and neutrophil activation. Clin Exp Immunol 2005; 140:470–7.

24. Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation and apoptosis. Clin Infect Dis 2005; 41(Suppl 7):S421–6.

25. Jones AQ, Bataki E, Elphick H, et al. Neutrophil survival is prolonged in the airways of healthy infants and infants with RSV bronchiolitis. Eur Respir J 2002; 20:651–7.

26. Lindemans CA, Coffer PJ, Schellens IMM, de Graaff PMA, Kimpen JLL, Koenderman L. Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. J Immunol 2006; 176:5529–37.

27. Esmann L, Idel C, Sarkar A, et al. Phagaocytosis of apoptotic cells by neutrophil granulocytes: diminished proinflammatory neutrophil functions in the presence of apoptotic cells. J Immunol 2010; 184: 391–400.

28. Boukhvalova MS. Respiratory syncytial virus infects and abortively replicates in the lungs in spite of pre-existing immunity. J Virol 2007; 81:9443–50.

29. Boukhvalova MS, Prince GA, Blanco JCG. The cotton rat model of respiratory viral infections. Biologicals 2009; 37:152–9.

30. Prince GA, Jenson AB, Hemming VG, et al. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. J Virol 1986; 57:721–8.

31. Rohwedder AKO, Forster J, Schneider K, Schneider E, Werchau H. Detection of RSV RNA in blood of neonates by PCR. J Med Virol 1998; 54:320–7.

32. Domurat F, Roberts NJ, Walsh EE, Dagan R. Respiratory syncytial virus infection of human mononuclear leukocytes in vitro and in vivo. J Infect Dis 1985; 152:895–902.

33. O'Donnell DR, McGarvey MJ, Tully JM, Balfour-Lynn IM, Openshaw PJ. Respiratory syncytial virus RNA in cells from the peripheral blood during acute infection. J Pediatr 1998; 133:272–4.

34. Yui I, Hoshi A, Shigeta Y, Takami T, Nakayama T. Detection of human respiratory syncytial virus sequences in peripheral blood mononuclear cells. J Med Virol 2003; 70:481–9.

35. Torres JP, Gomez AM, Khokhar S, et al. Respiratory syncytial virus (RSV) RNA loads in peripheral blood correlates with disease severity in mice. Respir Res 2010; 11:125.

36. Gu J, Xz Gao Z, Liu J, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007; 370:686–91.

37. Chan MC, Chan RW, Yu WC, et al. Influenza H5N1 virus infection of polarized human alveolar epithelial cells and lung microvascular endothelial cells. Respir Res 2009; 10:102.

38. Buckley CD, McGettrick HM, Osborne CE, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. J Leukocyte Biol 2006; 79:303–11.